AWARD NUMBER: W81XWH-13-1-0203

TITLE: Targeting Palmitoyl Acyltransferases in Mutant NRAS-Driven Melanoma

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REPORT DATE: August 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2014	Annual	1 Aug 2013 – 31 Jul 2014

4. TITLE AND SUBTITLE

Targeting Palmitoyl Acyltransferases in Mutant NRAS-Driven Melanoma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0203

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Massachusetts General Hospital
Boston, MA 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Mutations in N-RAS are linked to ~20% of melanoma with no effective treatment. Targeting palmitoyl acyltansferases (PATs) involved in N-RAS regulation could be a novel strategy to treat N-RAS mutant melanoma. The objective of the project is to identify PATs responsible for NRAS activation in melanoma cells using chemical biology and functional genomic approaches. In the first year of the study, we have developed more potent chemical probes to profile PATs in cells, and have carried out PATs profiling in melanoma cells using chemical probes and mRNA profiling. We have identified candidate PATs highly expressed in NRAS melanoma cells. We have developed shRNA reagents to further study their functions.

15. SUBJECT TERMS

NRAS, Melanoma, Palmitoylation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Mutations in N-RAS are linked to ~20% of melanoma with no effective treatment, representing a large unmet medical need. Palmitoylation (post-translational modification by adding a 16-carbon palmitate) is required for N-RAS oncogenic activity. Recently, 23 of DHHC (Asp-His-His-Cys)-family proteins were discovered as protein palmitoyl acyltransferases (PATs). Targeting DHHC-PATs involved in N-RAS regulation could be a novel strategy to treat N-RAS mutant melanoma. We have developed chemical probes that covalently label the active sites of DHHC-PATs, allowing us to investigate the enzymatic activities of PATs that are responsible for N-RAS palmitoylation using Activity-Based Protein Profiling (ABPP) approaches. The first objective is to identify DHHC-PATs regulating palmitoylation of oncogenic N-RAS in melanoma cells using ABPP methods and compare with the mRNA expression profile to identify candidate PATs highly active in NRAS mutant melanoma cells. The second objective is to evaluate the effects of shRNAs targeting DHHC-PATs on N-RAS activity, melanoma cell proliferation and apoptosis. Finally, we aim to design and synthesize analogues of cerulenin, a natural product inhibitor of PATs to identify small molecule inhibitors of N-RAS palmitoylation.

2. KEYWORDD

NRAS, melanoma, palmitoylation, DHHC domain containing palmitoyl acyltransferases, Activity based protein profiling (ABPP), proteomics

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goal of the project is to use chemical biology and functional genomics approaches to study palmitoyl acyltransferases involved in NRAS activation in melanoma cells. As we stated in the SOW of the grant, there are three major tasks of this project. Task1: To identify DHHC-PATs regulating palmitoylation of oncogenic N-RAS in melanoma cells (Month 1-12). Task2. To evaluate the effects of shRNAs targeting DHHC-PATs on NRAS activity, melanoma cell proliferation and apoptosis (Month 13-24). Task3. To design and synthesize analogues of cerulenin, a natural product inhibitor of palmitoyl acyltransferases to identify small molecule inhibitors of N-RAS palmitoylation. (in parallel with Task1 and 2, Month 0-24).

In the last funding period, as we stated in the SOW, our work is focused on Task 1 and we have initiated some work in Task 3. Below are the details of our activities regarding to the SOW.

What was accomplished under these goals?

Task 1a. Test the activity based probe (16C-BYA) for labeling efficiency in multiple N-RAS mutant melanoma cell lines (SK-Mel30, SK-Mel2, MEL-JUSO, BL, etc.) and control cell lines (BRAF mutant melanoma, A375, U62, MalMe, M14, SK-Mel5 and primary

human melanocytes) in 6-well format, optimize the concentration and labeling time (month 1-6).

Accomplishments: We have tested the labeling of the probe 16C-BYA in NRAS melanoma cells (SK-Mel30, SK-Mel2 and 501Mel), and compared the pattern of labeling with the control cell line A375, which carries BRAF mutation. The concentration and time of labeling has been optimized to 100uM of probe for 8 hours labeling. We have carried out Click chemistry using Biotin-Azide or Rhodamine-Azide, and have compared the labeling efficiency and pattern of the cell lines that we have studied. We found that the probe specifically labeled several bands in NRAS mutant cell lines, which might represent potential candidate palmitoyl acyltransferases regulating NRAS activities.

Task 1b. Proteomic and mass spectrometry analysis of labeled proteins (month 6-12).

Accomplishments: We have carried out large-scale labeling experiments under the oiptimized condition (100uM probe for 8hours labeling) using 5 dishes of 10cm dishes for SK-Mel2 and SK-Mel30 cells, which gave us sufficient proteins for follow-up proteomics studies. We carried out Click chemistry using Biotin-azide, and used Streptavidin bead to enrich the labeled protein. We carried out on-bead digestion using trysin and the peptides were submitted to Harvard Medical School Taplin Mass Spectrometry center for protein identification. We successfully finished the proteomics studies, and our studies suggested that 3 DHHC proteins were among the palmitoyl acyltransferases with high activities in the NRAS cell lines. These protein will be candidate PATs for further studies. We also carried out qRT-PCR studies of the NRAS mutant cell line (SK-Mel30) and BRAF mutant cell line (A375) of all 23 DHHC-palmitoyl acyltransferases. We developed qRT-PCR probes that allow us to detect all of them effectively. From our studies, we have found that 5 DHHC genes are highly expressed in the NRAS mutant cell lines. By comparing the mass spec results and the mRNA profiling, we have narrowed down to 2 candidate genes which are highly enriched in NRAS mutant melanoma.

Task 1c. Carry out bioinformatics studies of the expression profiles in a large set of melanoma cell lines from previously published datasets (month 3-6).

Accomplishments: We carried out bioinformatics studies by data mining of public available expression profiles. From NCBI GEO data bases, we have identified melanoma expression profiles. We found that DHHC4 and DHHC5 are among the highly expressed PATs in NRAS mutant cells. These results support some of our findings using qRT-PCR methods described above, but not necessarily be the only methods to provide candidate PATs.

Task 1d. Histology studies of PATs in melanoma cells to confirm that the protein levels of PATs are elevated in N-RAS mutant melanoma cell (month 6-12).

Accomplishments: We have studied the staining of a commercially available antibody for one of the top candidate PATs. However, this antibody did not recognize the correct protein in Western blot or immunoflurorescent staining. The antibody recognizes a different protein with a different size on the Western blot. The information showed on the vendor website is actually not correct. Therefore, there is no commercially available antibody could be used to study the protein level of the candidate PATs in histology. To address this issue, we will try to develop a new antibody that could recognize the

protein. Alternatively, we will look at the expression level of the candidate PATs using data mining and bioinformatics methods as well as qRT-PCRs using primary tumor samples from a tissue array. This effort is on going and will likely to accomplished in the second year of the study.

Task2. To evaluate the effects of shRNAs targeting DHHC-PATs on NRAS activity, melanoma cell proliferation and apoptosis (Month 13-24):

Task 2 will be carried out in the second year, and we will report our progress and findings in the next annual report.

Task3. To design and synthesize analogues of cerulenin, a natural product inhibitor of palmitoyl acyltransferases to identify small molecule inhibitors of N-RAS palmitoylation. (in parallel with Task1 and 2, Month 0-24):

Task 3a. Synthesize compound libraries based on the structure of cerulenin. Modifications of their head and tail groups might improve their selectivity and potency. ~50 compounds will be synthesized in 0-12 month.

Accomplishments: We have designed cerulenin-based chemical probes to study palmitoyl acyltransferases. Cerulenin is a natural product inhibitor of fatty acid biosynthesis and protein palmitoylation. It has been shown that a palmitoyl analogue for cerulenin has good selectivity over fatty acid synthases. We have synthesized an alkyne analogue of cerulenin and a synthetic strategy has been developed.

We then tested this probe in cells express HA-DHHC4. We found that this probe effectively labels DHHC proteins in doses lower than 2-BP based first-generation probes. More over, this probe labels protein in vitro in cell lystate, suggesting that live cell is not required for the labeling. This new probe will provide additional chemical tools to study palmitoylation and palmitoyl acyltransferases in melanoma cells. It will serve as a base to develop high throughput assays to identify small molecule inhibitors of palmitoyl acyltransferases.

Using the synthetic route that we developed in this task, we have synthesized several analogues of cerulenin with variations at the head groups. We have finished synthesis of ~10 compounds. More analogues are planned and will be made in the second year of studies.

What opportunities for training and professional development have the project provided?

The PI has strong background in chemistry and drug discovery. His career goal is to become a leading scientist and independent investigator in melanoma and skin cancer drug discovery. To achieve the career goal, the mentor (Dr. Fisher) has been guiding Dr. Wu's melanoma research through routine meetings and discussions. Dr. Wu has participated the MGH melanoma program weekly meeting chaired by Dr. Fisher. Dr. Wu has attended the melanoma workshops sponsored by Dana-Farber/Harvard Cancer Center (DF/HCC) and the Koch Institute of Cancer Research at MIT, which was organized by Dr. Fisher. These workshops covered melanoma clinical trials studies, melanoma genomics and drug discovery, and will provide additional training and

collaboration opportunities for young investigators. Dr. Wu has participated the melanoma journal club and seminars in MGH and Harvard community, where he will learn the progresses in melanoma research.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

As we planned in the original SOW, we will focus on Task 2 to fully validate the candidate PATs and their functions in melanoma. We will carry out shRNA knockdown experiments to show whether these PATs are essential for NRAS mutant melanoma proliferation and survival.

4. IMPACT

What was the impact on the development of the principle discipline of the project?

We have demonstrated that a chemical approach using Activity based protein profiling (ABPP) could study the palmitoyl acyltransferases in melanoma. We have developed multiple chemical tools to study PATs in cancer cells. Our study has identified candidate PATs possibly as new therapeutic targets, which could have significant impact in drug discovery and cancer research.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHNAGES/PROBLEMS

Changes in approach and reasons for change:

As we stated in the Accomplishment section, Task 1d of histology studies of PATs in the melanoma cells requires changes. The commercial antibody for the DHHC proteins failed to recognize the correct protein, and could not be used in histology studies. We

have changed to use qRT-PCR to evaluate the expression of the candidate PATs in melanoma samples, which will provide similar results.

6. PRODCUTS

Publications: A manuscript describing part of the work has been submitted to ***ACS Chemical Biology***, and is currently in revision.

"A Clickable Analogue of Cerulenin as Chemical Probe to Explore Protein Palmitoylation" Baohui Zheng, Shunying Zhu, and Xu Wu*, ***ACS chemical biology***, Submitted and under revision.

Acknowledgement of federal support: Yes

Other products:

Research material: chemical probes for palmitoyl acyltransferases (16C-BYA and 16-EYA)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Xu Wu
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-1624-0143
Nearest person month worked:	3
Contribution to project:	Dr. Wu has supervised the research, designed the experiments and interpreted the results
Funding support:	MGH Institutional fund American Cancer Society Melanoma Research Alliance National Cancer Institute

Name:	Baohui Zheng
Project Role:	Research Fellow

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to project:	Dr. Zheng has performed work in the area of synthesis, chemical labeling and mass spectrometry
Funding support:	Melanoma Research Alliance

Name:	Michael DeRan
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to project:	Dr. DeRan has performed work in the area of biochemistry, and mRNA expression profiling
Funding support:	MGH Institutional fund American Cancer Society National Cancer Institute

Has there been a change in the active other support of the PD/PI(s)?

A previously listed pending application has been awarded for the PI.

National Cancer Institute (NCI)/NIH, Wu (PI)

1R01CA187909-01

Metabolic regulation of cellular tight junction proteins

The goal of this proposal is to study the regulation of cellular junction proteins AMOTL1 by AMPK, and whether this regulation lead to inhibition of YAP oncogene in cancers in vitro and in vivo.

Overlap: None

This award will not impact the level of efforts for the DoD grant.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENT

N/A

9. APPENDICES

N/A